

Note

A 6×6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*

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Abstract

A protocol was developed using 96-well plates and multichannel pipettes for serial dilutions, followed by drop plating on agar in a 6 × 6 format. This protocol permits simultaneous plating of six dilutions which greatly decreases the number of plates utilized thereby saving incubator space for organisms such as *Campylobacter* which require unique environmental conditions. Published by Elsevier B.V.

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Two important approaches to bacterial enumeration are most probable number (MPN) and direct plating onto an agar-based medium (“pour”, “drop”, “spread”, and “spiral” plating). While the drop plate method is economical, and used in microbiology research laboratories worldwide, there is no standardized procedure for the size of the drops (10–30 µl per drop; Hoben and Somasegaran, 1982; Barbosa et al., 1995), the number of replications, or the number of sectors (dilutions) used per plate. In contrast, the pour, spread (or spiral) plating, and MPN techniques have standardized procedures for counting bacteria in a

wide variety of samples. Because spread/spiral plate methods involve the use of a large quantity of media (usually 3–5 plates per dilution), enumeration necessitates greater incubator space. Conventional MPN methods (de Man, 1983) are sensitive but not precise and the procedure is labor intensive. While numerous papers have compared the accuracy of various enumeration methods, most have only focused on direct plating techniques (Barbosa et al., 1995; Hedges et al., 1978; Snyder, 1947). Few papers have precisely compared the accuracy of direct plating to MPN (Sharpe et al., 1983). The aim of this work was to develop a modified drop plate scheme using standard 96-well plates for dilutions, combined with a 6 × 6 plating format to minimize the number of agar plates necessary for obtaining a precise bacterial enumeration. We have also shown that this format facilitates enumeration using simultaneous colony counting and MPN.

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Fig. 1 summarizes the 6×6 drop plate procedure. Briefly, 250 μL of sample was loaded into the first well of each row in a 96-well plate, and 10-fold serial dilutions were made using a multichannel pipette (Rainin, Emeryville, CA) by transferring 20 μL from column i into 180 μL of medium in column $\{i+1\}$, mixing 10 times, and repeating the process; pipette tips were changed between dilutions. Thereafter, six replicates of 10 μL (ideal for the spacing between the tips of a multichannel pipette) from each of the six selected dilutions were plated onto an agar medium using a multichannel pipette. Plates were allowed to dry, then placed into an incubator. Colonies (0.5–1 mm) were enumerated after an appropriate length of time. Depending on the bacteria species used, we adopted different incubation times and temperatures. *Escherichia coli* (poultry isolate) plates (Luria-Bertani; Difco, Detroit, MI) were incubated either at 26 °C for 16 h, or at 37 °C for 3 h, then at 22 °C for 16 h. *Listeria monocytogenes* ATCC 19115 plates (Brain-Heart-Infusion; Difco) were incubated at 37 °C for 24 h. *Campylobacter jejuni* (strain 81–176; Black et al., 1988) plates (Mueller-Hinton; Difco) were incubated at 42 °C for 24 to 30 h in microaerobic jars with CampyPak Plus (BBL, Becton-Dickinson, Sparks, MD).

To quantitatively illustrate the typical 6×6 drop plate method (Fig. 2; solid lines), overnight cultures of the three bacterial strains were diluted to give approximately 10^3 – 10^8 CFU ml^{-1} . At these concentrations, four 6×6 drop plates were utilized per sample ($n=24$). Serial dilutions of each sample were also prepared to yield approximately 50–200 CFU per 50 μL for spiral plating (Autoplate 4000, Spiral Biotech, Norwood, MA; $n=4$). The bacterial density obtained from spiral plating (\times), drop plate colony counting (\circ), and drop plate MPN (Δ) were plotted against the cell density estimated from the $n=96$, six-dilution MPN procedure (*E. coli* and *L. monocytogenes* only; Irwin et al., 2000). Details on the multiple dilution MPN calculation have been elaborated elsewhere (Irwin et al., 2001). Results from the drop plates of all three strains tested showed good agreement (slopes ~ 1) with the estimated cell density calculated from either the $n=96$, six-dilution MPN method (for *E. coli* and *L. monocytogenes*), or the starting culture diluted 10^5 -fold (for *C. jejuni*). For *C. jejuni*, the calculated cell concentration from the starting sample was used as the basis for comparison owing

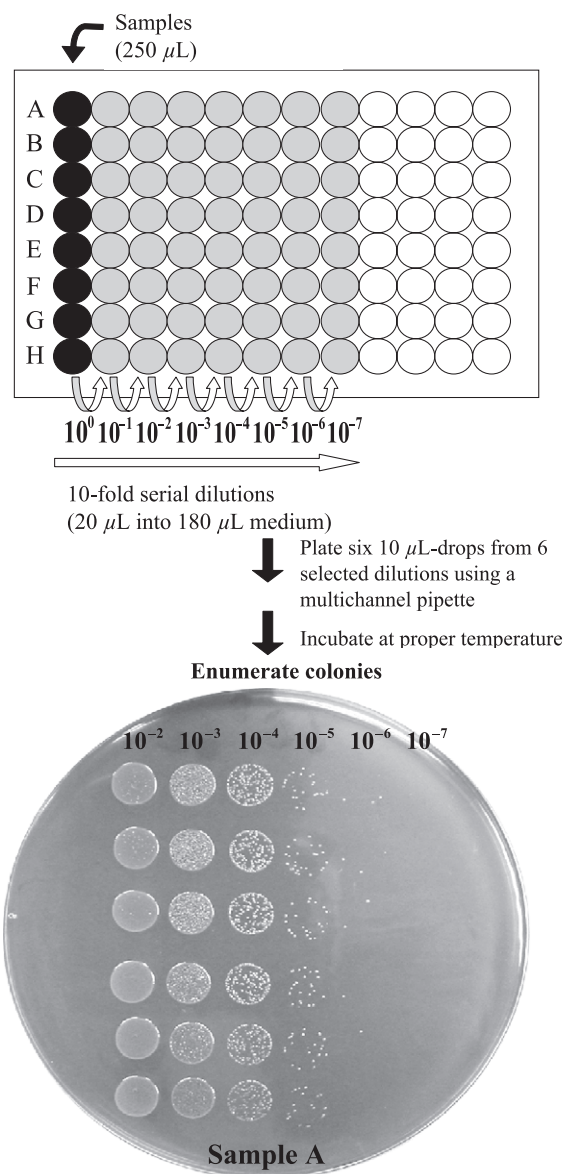


Fig. 1. Schematic of the 6×6 drop plate method. The bottom photograph depicts six drops \times six 1-to-10 serial dilutions of *C. jejuni*.

to the difficulty of obtaining discernable turbidity using the comparison MPN protocol. On the other hand, bacterial concentration derived from spiral plating showed greater deviation (in ca. 40% of all observations) from the comparison MPN than cell concentrations derived from either 6×6 drop plate colony counts, or 6×6 drop plate MPN.

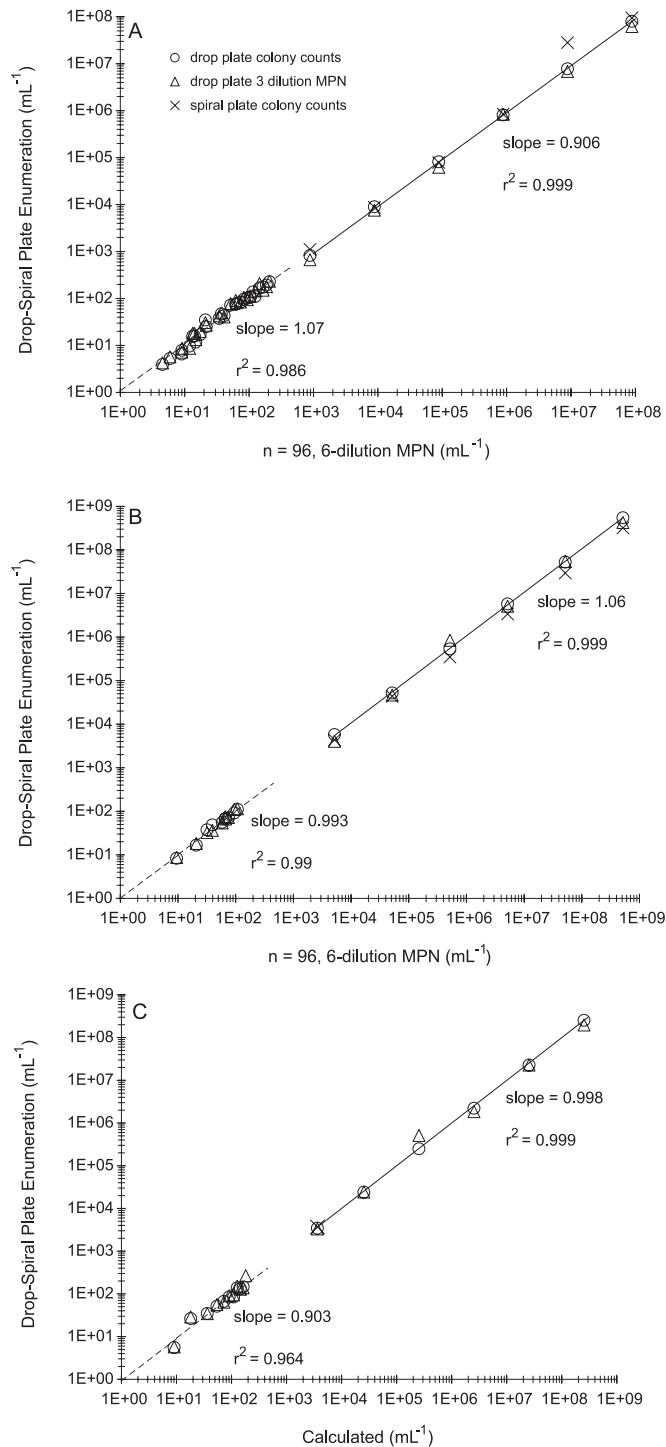


Fig. 2. Correlation of colony count cell density and MPN with estimated cell concentrations. Solid lines represent least square fits for the standard 6×6 drop plate method ($\geq 10^3$ CFU mL^{-1} ; $n = 24$). Dashed lines correspond to fits for the single-dilution 6×6 drop plate method (≤ 200 CFU mL^{-1} ; $n = 72$). (A) *E. coli*; (B) *L. monocytogenes*; (C) *C. jejuni*. \circ , drop plate colony counts; Δ , drop plate MPN; \times , spiral plate colony counts.

When a low concentration of organisms (≤ 200 CFU ml $^{-1}$; Fig. 2, dashed lines) was expected, the above procedure was modified: 4 ml of each sample was poured into a sterile solution basin (Labcor Products, Frederick, MD) and directly plated using the 6×6 format onto two agar plates ($n=72$ observations). At these concentrations, the single-dilution MPN equation was utilized (Halvorson and Ziegler, 1933)

$$\text{MPN ml}^{-1} = -\frac{\text{Ln}\left[\frac{n-p}{n}\right]}{v}$$

whereupon $n=72$, p is the number of positive responses, and v is the volume plated (10 μ l) per drop. All three species tested showed good agreement with the observed ($n=96$, six-dilution MPN) or estimated cell density (slopes of 1.07, 0.993, and 0.903; r^2 of 0.96–0.99). The limit of detection was estimated to be 7 for *E. coli*, 8 for *L. monocytogenes*, and 18 CFU ml $^{-1}$ for *C. jejuni*. These results are similar to the comparison MPN technique and argue that our approach is nearly as sensitive, more economical (70% savings in

supplies), and less laborious (80% savings in time) than the $n=96$, six-dilution MPN scheme.

Because of concerns over potential sampling error associated with small volumes (10 μ l), we have performed a set of colony distribution experiments exploiting the single-dilution drop plate method (10 μ l per drop; $n=180$). Fig. 3 shows the Poisson (discrete) distribution of colony count frequency of occurrence (per 10 μ l) curve fits (bold grey lines; Irwin et al., 1994) relative to the observed data (symbols and dashed lines). These data demonstrate that both observed (\bar{C} , s) and theoretical (μ , σ) averages and standard deviations agree well (average deviation of 14%). Therefore, the distribution of colonies per drop obeys binomial probability theory (Student, 1907) and suggests that sampling errors were negligible.

In summary, the 6×6 drop plate method provides excellent accuracy, sensitivity and precision for enumeration of a wide range of bacterial concentrations and concurrently affords savings in sample processing

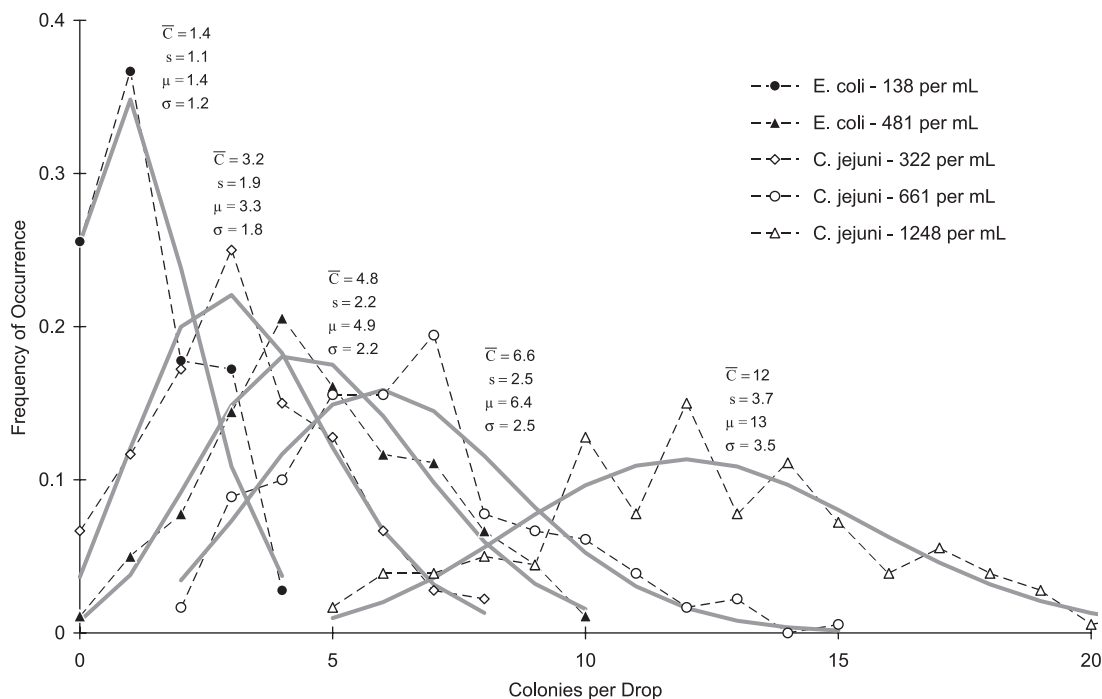


Fig. 3. Theoretical (bold, grey lines) and observed (●, 138; ◇, 322; ▲, 481; ○, 661; △, 1248 CFU ml $^{-1}$) frequency of occurrence of each colony count C_i per drop. In our usage, s is the observed standard deviation and \bar{C} is the average observed colony count per drop. The theoretical standard deviation $\sigma=\sqrt{\mu}$ (Student, 1907) whereupon μ is the estimated population average colony count per drop based upon nonlinear regression fit of the observed frequencies with respect to the Poisson distribution $\text{Exp}[-\mu]\mu^{C_i}/C_i!$.

time, material costs, and conservation of incubator space. This protocol also has extra utility as one can use the same data to obtain an MPN. Thus, the drop plate MPN can be exploited either as an internal control to verify the plate count, or as an estimate of cell density without concomitant colony counting.

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